A PYROPHOSPHATE BASED ENERGY GENERATING MODULE

James P. Chambers, Ph.D.*
The University of Texas at San Antonio
San Antonio, TX 78249

Jennifer Sekowski, Ph.D.
US Army RDECOM
Aberdeen Proving Ground, MD 21010

James J. Valdes, Ph.D.
US Army RDECOM
Aberdeen Proving Ground, MD 21010

1. ABSTRACT

The most widely used transient form of stored energy in biological systems is the complex, organic molecule, adenosine triphosphate (ATP). However, the much smaller, simpler pyrophosphate (PP_i) molecule could potentially serve as an inexpensive, high energy bond, ATP mimic ($PP_i \rightarrow 2\ P_i$, $\Delta G^{0'}$ = -33 Kcal/mol). Pyruvate Phosphate Dikinase (PPDK), an enzyme found primarily in a small number of *primitive protists* reversibly transfers phosphate (P_i) from phosphoenolpyruvate (PEP) and PP_i to AMP producing ATP. Using a PPDK gene construct, recombinant PPDK (*r*PPDK) was expressed in *E. coli*, purified on nickel(II) coupled sepharose, and characterized by PAGE analysis (M_r = 115,000). Kinetic analysis revealed *1)* µM Km values for PEP, PP_i, and AMP and *2)* a k_{cat} value (turnover number) of 1.0 x 10^2. Alpha-Hemolysin pore-functionalized, lipid bilayers facilitated movement of PEP and PP_i through 1-2 nm pores with concomitant Luciferase coupled ATP-dependent generation of light. This is an important first step towards developing self-renewing power for nano/micro systems.

2. INTRODUCTION

Significant advances in systems biology (genomics, proteomics, metabolomics) have made possible contemplation of development of responsive, artificial nano/micro chemical networks, *i.e*., acellular, metabolic circuits/constructs/mimics capable of carrying out in orchestrated fashion biochemical reaction cascades which could give the war-fighter significant added advantage, *i.e*., capabilities (awareness, response and adaptation) for dealing with emerging threats that may affect in very negative fashion force operational capabilities. However, these responsive nano/micro chemical networks must be provided a simple, self-renewable power/energy source. Currently, there are no small scale renewable energy sources available.

Living systems have developed over the eons very efficient but complicated means for extraction of energy. The most widely used high-energy compound (phosphoric acid anhydride) is adenosine triphosphate (ATP → ADP + Pi, $\Delta G^{0'}$ = -30.5 kJ/mol); a large, complex molecule consisting of a nitrogenous base (adenine) and ribose phosphate. The most important parts of the molecule with regard to energetics are the two phosphoric acid anhydrides (γ and β phosphates).

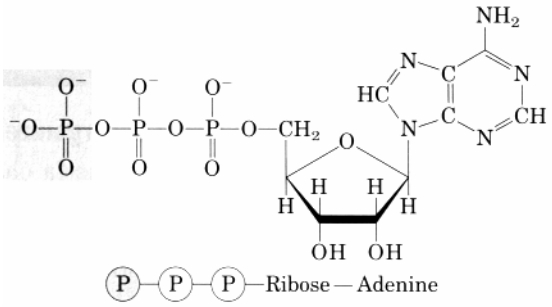

Figure 1: Structure of ATP

In reciprocating fashion, ADP can accept both phosphate and energy and the ATP thus formed can donate phosphate and energy. Thus, the ATP/ADP donor/acceptor pair is a transient energy shuttle carried from point to point, from one enzyme system to another. Most importantly, when coupled to ATP-substrate specific enzymes catalyzing thermodynamically unfavorable reactions, a 100 to 200 million fold increased equilibrium ratio of products to starting reactants is achieved independent of the nature of the reaction.

The primary source of energy, *i.e*., ATP for amitochondriate organisms is glycolysis, a multi (10) enzyme metabolic process resulting in the stepwise degradation of glucose and other simple sugars. Glycolysis is a paradigm of metabolic pathways. Carried out in the cytosol of cells, it is basically an anaerobic process; its principal steps occur with no requirement for oxygen. Thus; living entities first appeared in an environment lacking O_2, and glycolysis was an early and important pathway for extracting energy from nutrient molecules.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A Pyrophosphate Based Energy Generating Module	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **The University of Texas at San Antonio San Antonio, TX 78249**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008, The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**6**	

Glycolysis played a central role in anaerobic metabolic extraction of energy during the first 2 billion years of biological evolution on earth. Modern organisms still employ glycolysis to provide precursor molecules for aerobic catabolic pathways such as the tricarboxylic acid and as a short-term energy source when oxygen is limiting.

Interestingly, the glycolytic process requires priming, i.e., input of energy in the form of ATP (the first reaction, *i.e.*, Hexokinase catalyzed phosphorylation of glucose and third reaction, *i.e.,* the Phosphofructokinase catalyzed phosphosphorylation of Fructose-6-Phosphate (*cf.* Figure 2). The hydrolysis of ATP to ADP is coupled with these two reactions. The energy generation reactions of glycolysis involve 2 substrate phosphorylation reactions (ADP + Pi → ATP) mediated by the enzymes Glycerate Kinase and Pyruvate Kinase, respectively. There is one oxidation step in Glycolysis, *i.e.*, the 3-Phosphoglyceraldehyde Dehydrogenase oxidation of 3-Phosphoglyceraldehye with concurrent conversion of NAD^+ →NADH giving rise to a high energy mixed anhydride, *i.e.*, 1,3-Diphosphoglyceric Acid. However, it does not involve molecular oxygen. The complete series of reactions is shown in Figure 2.

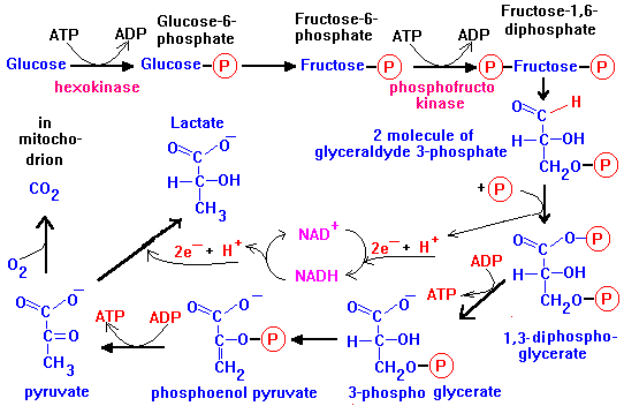

Figure 2: Summary of glycolysis.

In contrast, energy derived from the oxidation of metabolic fuels in aerobic, *i.e.*, mitochrondriate eukaryotes is ultimately converted to ATP via the electron transport scheme embedded in the inner mitochondrial membrane. In this process (summarized in Figure 3), electrons are transferred from NADH or $FADH_2$ to molecular oxygen (O_2) by a series of membrane embedded protein complexes (I-IV) giving rise to a proton gradient. The reverse flow of protons back through the membrane bound F_1-ATP Synthase (Complex V) into the mitochondrial matrix drives the formation of approximately 95 % of the total ATP used by eukaryote cells.

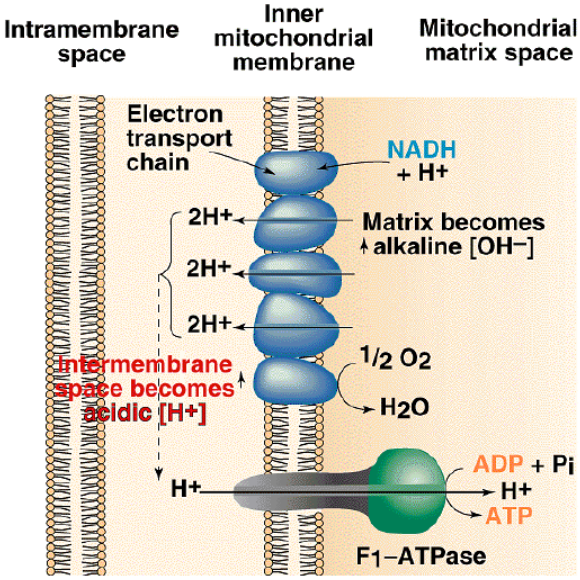

Figure 3: Coupling of electron transfer and ADP phosphorylation.

Like ATP, the much smaller and simpler pyrophosphate molecule (PPi, structure shown in Figure 4) readily goes toward equilibrium, *i.e.,* PPi → Pi + Pi ($\Delta G^{0'}$ = -33.6 kJ/mol). Pyrophosphate is formed in a host of synthetic reactions including the activation of amino acids by Aminoacyl-*t*RNA Synthetases, activation of fatty acids by Thiokinases to form CoA esters, activation of carbohydrates by Uridyl Transferases, and formation of nucleic acids by Polymerases.

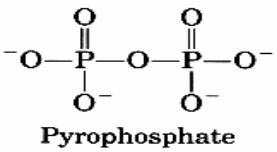

Figure 4: Structure of pyrophosphate.

The accepted dogma has been that the energy of the pyrophosphate anhydride is not used. However, due to its energy equivalence (possesses the identical phosphoric acid anhydride motif found in ATP), pyrophosphate qualifies as a plausible, inexpensive energy source for ATP synthesis. Importantly, a simple, efficient, means of energy extraction makes many nano/micro 'acellular' applications readily available using a very large and diverse repertoire of ATP-dependent

enzymes/proteins of significant value in the short term.

Borrowing naturally occurring structural and chemical themes, we propose to configure in stable boundary structures, *i.e*., liposomes, bead immobilized conjugates, etc., two simple reactions which when coupled results in robust generation of ATP using PPi as energy source. The two reactions are:

Reaction 1:

Oxaloacetate + PPi → P-enolpyruvate + CO_2 + Pi

Reaction 2:

P-enolpyruvate + AMP + PPi → Pyruvate + ATP + Pi

Reaction 1 in which PPi is used in place of ATP as a phosphorylating agent was discovered in *Propionibacterium shermanii* and is catalyzed by the enzyme Carboxytransphosphorylase E.C.4.1.1.38 (CTP). Reaction 2 originally described in *Bacillus symbiosis* and *Entamoeba histolytica* is catalyzed by the enzyme Pyruvate Phosphate Dikinase E.C.2.7.9.1 (PPDK) with PPi serving as direct energy source for formation of ATP. Thus, Reaction 1 provides the phosphorylated keto acid, *i.e*., P-enolpyruvate which is utilized in Reaction 2, the PPDK catalyzed reaction.

Work presented in this report describes expression of a carboxyterminal histidine tag containing PPDK gene construct from *E. histolytica* in *E. coli*. Expressed recombinant PPDK (*r*PPDK) was purified on nickel coupled sepharose columns, and characterized by PAGE analysis (Mr = 115,000 Da). Kinetic analysis revealed µM Km values for PEP, PPi and AMP. *r*PPDK exhibited a k_{cat} (turnover) value of 1.0 x 10^2 sec^{-1}. Although not yet coupled to the CTP enzyme (currently being cloned), preliminary experiments using lipid coated Transwell filter membranes made porous with insertion of α-Hemolysin, a pore forming protein indicate partitioned *r*PPDK ATP synthesis continued in the absence of added AMP and PPi when coupled to ATP-dependent Luciferase generation of AMP and PPi in the presence of PEP. The functionalized Transwell membranes represent an important starting point in successful partitioning of these enzymes within complex substrate permeable boundary structures, *i.e*., liposomes.

Our ultimate goal is to develop a PPi/Pi, ATP-*independent*, donor/acceptor chemistry for energy scavenging, production and storage using dendrimers, *i.e*., artificial donor/acceptor scaffolds. This work represents a first and important step towards achieving that goal which has great utility for the war-fighter.

3.0 EXPERIMENTAL METHODS

1.0 Expression of *r*PPDK

Expression was carried out according to modification of the procedure of Saavedra-Lira and coworkers [1]. The amplified fragment was initially cloned as a *Nhe*I fragment in pET23b expression vector which allowed expression of the PPDK enzyme fused to a carboxyterminal sequence of polyHis in *E. coli* strain Rosetta-gami. High efficiency transformation of *E. coli* was achieved by electroporation [2]. Selection was achieved by transformant inoculation of LB medium containing 100 µg/ml ampicillin incubated at 37 ^{0}C with shaking. The culture was grown to an $O.D._{600\ nm}$ of 1.0 at which time 0.4 mM IPTG was added and incubation continued for 14 hr. Cells over expressing *r*PPDK were centrifuged (5000 x g, 5 min) and the pellet resuspended in imidazole buffer (5 mM, pH 7.9) containing 50 mM NaCl and 20 mM Tris-HCl. Suspended cells were lysed using a French press, cell debris removed by centrifugation (39000 x g, 20 min), and resulting cell lysate supernatant served as source of enzyme for purification. Cell lysate supernatant was passed through a 0.2 µ sterile filter, aliquoted, and stored at 22 ^{0}C. Protein content was determined using the method of Bradford [3].

2.0 *r*PPDK and Kinetic Analysis

Enzyme purification was achieved using a 1 ml Hi-Trap column (Pharmacia) coupled with Ni^{2+} previously washed with 2 volumes 0.1 M $NiSO_4$, 5 volumes water and 10 volumes imidazole buffer (5 mM, pH 7.9). Cell lysate supernatant was applied to the affinity column and washed with 15 columns imidazole buffer (60 mM, pH 8.1) and *r*PPDK enzyme eluted with 5 volumes imidazole buffer (100 mM, pH 8.2). Cell lysate and purified enzyme was stored at 22 ^{0}C. *r*PPDK was assayed using the ATP-Dependent Luciferase assay. The relationship between ATP concentration and light intensity is based upon the Michaelis-Menten Equation in the following form:

$$\text{Light Intensity} = (V_{max} \times C_{ATP})/(K_m + C_{ATP})$$

At low ATP concentrations (C_{ATP}<<K_m), the formula reduces to light intensity = V_{max} x C_{ATP}/K_m. Thus, light output is directly proportional to the ATP concentration (C_{ATP}) and dependent on the amount of luciferase (V_{max}) present. ATP produced was directly extrapolated by use of a standard curve shown in Figure 5. The Michaelis constant (K_m) was derived for each substrate (PPi, PEP, and AMP) using double reciprocal Lineweaver-Burk plots of saturation data.

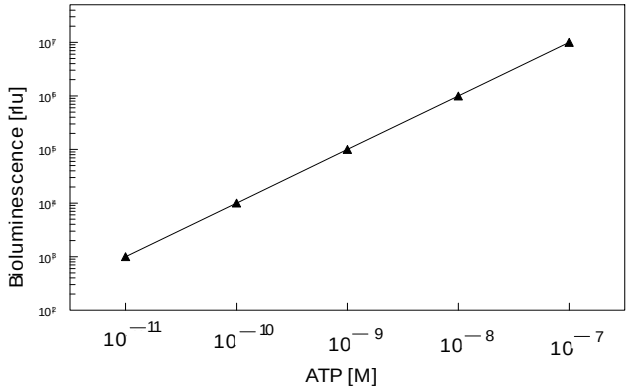

Figure 5: ATP Standard Curve.

ATP standards were obtained by serial dilution and bioluminescence determined using a BioTek Synergy 2 Luminometer.

3.0 Transwell Bilayer Partitioned *r*PPDK-Luciferase Coupled Reactions

Asolectin membranes were formed across 0.4 µ diameter Transwell (Corning) filter pores according to the procedure of Kasianowicz [5]. Bilayers were made with/without added (0.25 µg) pore forming α-Hemolysin [5]. As shown in Figure 6, Transwell filters consist of an upper (0.1 ml) and lower (0.6 ml) reservoir separated by a porous membrane (0.4 µ diameter pores, 1.3 x 10^6 pores/0.33 cm^2).

Luciferase from *Photinus pyralis* (American firefly) catalyzes the following reaction:

$$ATP + \text{D-luciferin} + O_2 \rightarrow \text{oxyluciferin} + PP_i + AMP + CO_2 + \text{light}$$

*r*PPDK catalyzes the following reaction:

$$AMP + PP_i + PEP \rightarrow ATP + \text{Pyruvate}$$

Thus, the product of the *r*PPDK reaction (ATP) is the substrate for the Luciferase/Luciferin reaction which produces two products, i.e., substrates AMP and PPi which are utilized by the *r*PPDK enzyme. PEP and PPi substrates were placed in the upper reservoir; whereas, AMP, D-Luciferin, lysate supernatant containing *r*PPDK and Luciferase were placed in the lower reservoir. Following a 2 hr equilibration period, an aliquot of the lower reservoir was removed and bioluminescence determined using the ATP-dependent Luciferase/Luciferin assay as previously described.

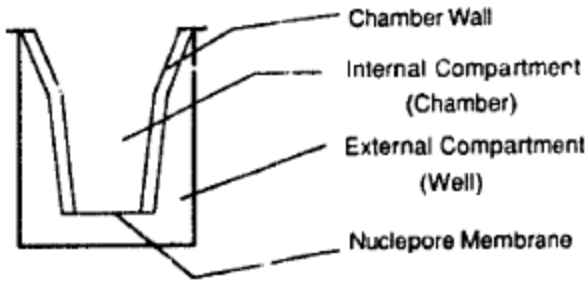

Figure 6: Diagram of the Transwell Filter System.

4.0 RESULTS

Shown in Figure 7 are double reciprocal plots for *r*PPDK substrates, PPi, PEP, and AMP. Extrapolation to the 1/X axis (-1/K_m) indicated a micromolar Michaelis constant (K_m) for the respective substrates. Summarized in Table 1 are purified *r*PPDK kinetic analysis and purification data.

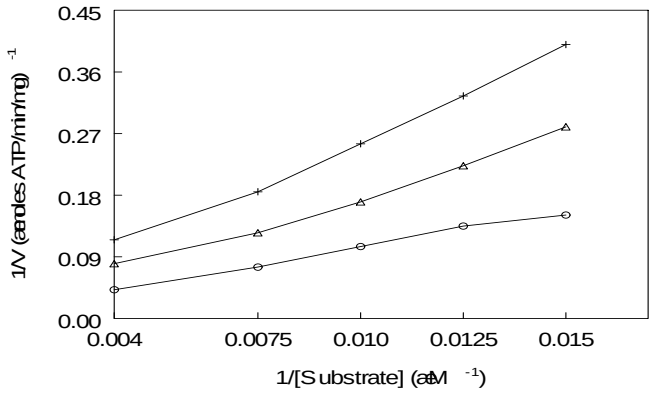

Figure 7: Lineweaver-Burk Plots of Saturation Data.

+ = PPi; open triangles = PEP; open circles = AMP.

Table 1: *r*PPDK Purification Data and Kinetic Analysis

Purification Data

Step	Total Protein (mg)	Total Activity µmoles ATP/min	Specific Activity µmoles ATP/min/mg	Yield	Fold Purification
Supernatant	14.5	11.2	0.77	100	1
100 mM eluate	1.1	10.1	9.2	90	11.9

Michaelis Constant (K_m)

PP_i (µM)	75
PEP (µM)	40
AMP (µM)	25

Table 1: *r*PPDK activity (1 µl, supernatant/eluate diluted 1:200) was assayed using the ATP-Dependent Luciferase/Luciferin Assay as described above using a BioTek Synergy 2 Luminometer. ATP was quantitated using the standard curve shown in Figure 5. The Michaelis constant (K_m) was determined keeping two of the three substrate concentrations constant (mM) and varying only one.

Using lipid coated Transwell filter membranes 0.4 µ diameter artificial lipid bilayers were made using azolectin [4] with/without added (0.25 µg) pore forming α-Hemolysin [5]. As shown in Table 2, PPi and PEP moved freely by diffusion from the upper to lower reservoir in the absence of lipid bilayer. Lipid bilayer covered Transwell filter pores when made porous with α-Hemolysin apparently formed pores allowing passage of PPi and PEP. In the absence of α-Hemolysin, some substrate (~13 %) moved into the lower reservoir as evidenced by the generation of light indicating some leakage. Thus, pore functionalized membranes (α-Hemolysin) allowed passage of PEP and PPi resulting in synthesis of ATP and generation of light via coupled luciferase/*r*PPDK.

Table 2: Transwell Filter Bilayer Partitioning

	1	2	3	4
Bilayer	-	+	+	+
α-Hemolysin	-	-	+	+
Upper Reservoir	+PP_i, +PEP	+PPi, +PEP	+PPi, +PEP	+PPi, +PEP, +Luc
Lower Reservoir	+AMP, +*r*PPDK, +DLuc, +Luc	+AMP, +*r*PPDK, +DLuc + Luc	+AMP, +*r*PPDK, +DLuc +Luc	+AMP, +*r*PPDK, +DLuc -Luc
Chemiluminescence[1]	38 nmoles	4.9 nmoles	30.0 nmoles	0 nmoles

Table 2: PPi, PEP, and AMP were added to the respective reservoirs at a final concentration of 400 µM. Luciferin/Luciferase reagent (50 µl) was added to 550 µl reaction buffer containing 50 µgms *r*PPDK(lower reservoir). A solvent free layer of asolectin liposomes was prepared according to the procedure of Montal and Mueller [4] as modified by Takagi and coworkers [6]. Condensed films of asolectin (0.5% v/v) dissolved in cholesterol:chloroform (2.5:1) were painted onto the upper and lower filter surfaces at room temperature. Filters were air dried and the lower reservoir buffer +/- α-Hemolysin (0.25 ngm) carefully increased to touch the bottom of the filter surface. The upper reservoir was treated in similar fashion the only exception being the layering of the upper buffer onto a thin layer of chlolesterol:chloroform (2.5:1) with removal of the formed meniscus by careful aspiration. Buffer containing PPi , PEP and +/- α-Hemolysin (0.25 ngm) was added drop wise to the upper reservoir being very careful to not disturb the surface adhered monolayer. Aliquots (50 µl) of the respective reaction mixtures (bottom reservoir) were removed 2 hr following addition of PPi and PEP to the upper reservoir. Bioluminescence was measured using a BioTek Synergy 2 Luminometer. DLuc = D-Luciferin; Luc = Luciferase. [1]Total chemiluminescence lower reservoir.

5.0 CONCLUSIONS

Recombinant PPDK enzyme was *1)* successfully expressed in *E. coli* and purified on Ni^{2+} coupled Sepharose, *2)* shown to generate ATP in the presence of substrates AMP, PEP, and PPi, *3)* exhibited high affinity for all substrates (micromolar), *4)* exhibited a turnover number (k_{cat}) of 1.0 x 10^2 sec^{-1}, and *5) r*PPDK generated ATP was coupled to ATP-dependent Luciferase mediated generation of light. Thus, microgram quantities of *r*PPDK (10^{-13} moles) turning over ~ 100 ATP molecules per sec, would correspond to ~10^{12}-10^{13} ATP molecules per sec total provided micromolar quantities of substrates are provided.

Inclusion of pore forming α-Hemolysin was shown to facilitate passage of both PEP and PP_i through lipid bilayer covered Transwell filter pores demonstrating facilitated permeability and partitioning. Asolectin was used initially because of the high percentage (approximately 30 %) of positively charged phosphatidylcholine.in concert with the slight negative charge to the surface of the filter. Although the lipid bilayer Transwell filter configuration is simple and results encouraging, the formed lipid mono-/bilayer moiety will require continued characterization and optimization. Regardless, this approach does provide a means to pursue partitioning of this catalytic function in a wide variety of membranous structures.

6.0 ACKNOWLEDGEMENTS

This work was supported by the Defense Threat Reduction Agency-Joint Science and Technology Office, Basic and Supporting Sciences Division and Army Contract W911SR-08-C-0024.

7.0 REFERENCES

1. Saavedra-Lira, E., Ramirez-Silva, L., and Perez-Montfort, R. *Biochimica et Biophysica Acta* 1382, 47-54 (1997).

2. Dower, William J., BioRad Laboratories.

3. Bradford, M. *Anal. Biochem*. 72, 248-256 (1976).

4. Montal, M. and Mueller, P. *Proc. Natl. Acad. Sci. USA* 62, 3561-3566 (1972).

5. Kasianowicz, J. J., Brandin, E., Branton, D., and Deamer, D. W. *Proc. Natl. Acad. Sci. USA* 93, 13770-13773 (1996).

6.. Takagi, M. (1967) *in* Experimental Techniques in Biomembrane Research, ed., Ohnishi, T. (Nankodo Publ. Co., Ltd., Tokyo), 385-392.